Environ. Sci. Technol. **2005**, *39*, 6681−6691

Metabolites Detected during Biodegradation of $^{13}C_6$-Benzene in Nitrate-Reducing and Methanogenic Enrichment Cultures

ANIA C. ULRICH,† HARRY R. BELLER,‡ AND ELIZABETH A. EDWARDS*

Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, M5S 3E5, Canada

The mechanism for anaerobic metabolism of benzene remains unknown. To date, there have been only a few studies reporting metabolites of anaerobic benzene biodegradation, in part because anaerobic benzene-degrading enrichment cultures are not very common and only two isolates have been characterized to date. Phenol and benzoate, metabolites consistent with benzene hydroxylation or benzene carboxylation, have been identified previously in mixed cultures, and more recently benzene methylation to toluene has been proposed as another possible mechanism for anaerobic benzene degradation. In this study, $^{13}C_6$-benzene was added to nitrate-reducing and methanogenic enrichment cultures and specific ^{13}C-labeled metabolites were monitored over time. The putative metabolites were detected by gas chromatography/mass spectrometry in ether extracts of 100-mL samples of culture taken at each time point. This method of analysis provided the sensitivity required to accurately quantify low concentrations of these compounds. In addition, benzoate trapping was used in an attempt to increase concentrations of upstream metabolites. In both cultures, in the presence and absence of unlabeled benzoate (trap), [*ring*-^{13}C]-toluene and [*ring*-^{13}C]benzoate were detected transiently during degradation. The data strongly support initial methylation of benzene to toluene, followed by transformation to benzoate. Although benzene methylation has been proposed previously, this is the first direct evidence to support this pathway. In the methanogenic culture only, $^{13}C_6$-phenol was also detected. The transient appearance of phenol, which appeared to be further transformed to benzoate, suggests that a pathway involving hydroxylation to phenol, as proposed in other studies, was also operative.

Introduction

Petroleum products are widely used for motive power, lubrication, fuel, dyes, and industrial solvents. They frequently contaminate soil and groundwater from inadvertent spills, leaking underground storage tanks, and seepage from waste disposal sites. Approximately 35% of the 1.4 million gasoline storage tanks in the United States are leaking (*1*). The petroleum products that cause the most serious groundwater pollution are the aromatic hydrocarbons benzene, toluene, ethylbenzene, and xylenes, collectively known as BTEX. Groundwater contamination by benzene is of particular concern, since benzene is the most water soluble of the BTEX compounds and is carcinogenic (*2*, *3*).

Microbial degradation of benzene is an important mechanism for natural attenuation (*4*−*6*). Although benzene is susceptible to other mechanisms of natural attenuation, such as sorption, volatilization, and dilution, biodegradation by indigenous microorganisms is the only attenuation mechanism resulting in destruction of the contaminant. It is well established that benzene can be metabolized under aerobic conditions; benzene is degraded to catechol via a dioxygenase-catalyzed reaction (*7*, *8*); benzene can also be degraded to phenol by monooxygenase-catalyzed reactions (*48*). These intermediate compounds are ultimately degraded to carbon dioxide. All of the BTEX compounds can also be biodegraded under anaerobic conditions, although benzene-degrading cultures have been the most difficult to cultivate in the laboratory. Biodegradation of benzene has been shown to occur under methanogenic (*9*−*11*), nitrate-reducing (*8*, *12*, *13*), iron-reducing (*9*, *14*−*17*), and sulfate-reducing (*9*, *18*−*24*) conditions. Anaerobic biodegradation is of particular significance, since BTEX-contaminated groundwater becomes anaerobic as oxygen is depleted in the early stages of biodegradation.

The importance of anaerobic processes in natural attenuation of hydrocarbons has been enhanced by the discovery of novel mechanisms for anaerobic oxidation of TEX compounds and alkanes. Most notably, a recently discovered class of synthase (specifically, fumarate addition) reactions involving the addition of a methyl group from toluene, xylene, or an alkane across the double bond in fumarate has now been shown to be widely employed by diverse microbes to activate these molecules in the absence of oxygen (*25*, *26*). However, such a strategy is difficult to envision for benzene, which has no methyl group. Naphthalene, also an unsubstituted aromatic compound, is thought to be activated via carboxylation (*27*, *28*). The anaerobic metabolism of oxidized aromatic compounds such as benzoate and phenol occurs readily and is relatively well understood (*29*). In fact, it appears that benzoate (or its CoA thioester) is a central intermediate in the anaerobic biodegradation of aromatic compounds (*29*).

There have been only a few studies documenting metabolites of anaerobic benzene degradation. Three mechanisms for the initial steps of the degradation of benzene have been proposed: (1) benzene hydroxylation to form phenol, (2) benzene carboxylation to form benzoate, and (3) benzene methylation to form toluene. In the earliest study describing metabolites of anaerobic benzene degradation, Vogel and Grbić-Galić (*30*) demonstrated that oxygen derived from water was incorporated into benzene to yield phenol in a methanogenic enrichment culture. Caldwell and Suflita's (*31*) observations were the first to implicate benzoate as an intermediate of benzene degradation in methanogenic, sulfate-reducing, and iron-reducing cultures. As a result, direct benzene carboxylation to benzoate was proposed as the first step in metabolism. Coates et al. (*32*) proposed that the initial step in anaerobic benzene degradation is alkylation of benzene to toluene, although no direct evidence was provided to support this mechanism. Rather, they showed that benzene degradation was inhibited by the addition of putative metabolites toluene, benzoate, benzaldehyde, benzyl

* Corresponding author phone (416)946-3506; fax: (416)978-8605; e-mail: edwards@chem-eng.utoronto.ca.

† Golder Associates Ltd., 2390 Argentia Road, Mississauga, Ontario L5N 5Z7, Canada.

‡ Lawrence Livermore National Laboratory, P.O. Box 808, L-542, Livermore, CA 94551.

10.1021/es050294u CCC: $30.25
Published on Web 07/21/2005

alcohol, and acetate, while catechol, phenol, cyclohexane, benzylsuccinate, or fumarate had no effect. Also, benzene degradation was stimulated by vitamin B_{12} addition and was inhibited by propyl iodide, an inhibitor of cobalamin-mediated methylation reactions.

Here, we describe the results of experiments using $^{13}C_6$-benzene to identify ^{13}C-metabolites during anaerobic benzene biodegradation in two distinct enrichment cultures, one methanogenic and one nitrate-reducing. Isotope trapping was used to enhance the concentrations of metabolites and has been used previously for anaerobic toluene and benzene degradation studies (*10*, *11*, *19*). The labeled substrate provides an indisputable link between the substrate and any products detected. The addition of unlabeled putative metabolite (the trap), in this case benzoate, should result in a buildup of ^{13}C-labeled benzoate and upstream labeled metabolites, provided that benzoate is indeed a metabolite and that the added benzoate can enter the cell and effectively dilute the metabolite pool.

Experimental Section

Enrichment Cultures. A methanogenic enrichment culture was derived from microcosms originally prepared with soil and groundwater from an oil refinery in Oklahoma (*33*, *34*). A nitrate-reducing enrichment culture was derived from microcosms prepared with soil and groundwater from a decommissioned retail gasoline station on Cartwright Avenue in Toronto, Ontario (*33*, *34*). Over the past eight years, these enrichment cultures have been successively transferred into defined mineral medium (*34*) and amended repeatedly with benzene (from a neat stock) ranging in concentration from 130 μM to 1100 μM. The nitrate-reducing enrichment cultures were also amended with sodium nitrate (2−5 mM final concentration from a 500 mM anaerobic stock) as required. The microbiology and kinetics of these enrichment cultures were described in more detail previously (*34*).

Toluene Degradation Experiments. In these experiments, benzene-degrading enrichment cultures were tested for their ability to also degrade toluene. For each enrichment culture, 300 mL of culture was centrifuged (2000*g* for 10 min), the supernatant was discarded, and the pellet was resuspended in a total of 600 mL fresh defined mineral medium. This 600 mL was then divided into six replicate 100-mL portions in 250-mL glass bottles sealed with screwcap Mininert (Supelco Inc.) valves. Three of the methanogenic replicate bottles were amended with 300 μM toluene, while the other three replicates were amended with 380 μM benzene. Similarly, three of the nitrate-reducing replicate bottles were amended with 100 μM toluene and 2 mM $NaNO_3$, while the three other replicates were amended with 170 μM benzene and 2 mM $NaNO_3$. The concentrations of toluene and benzene are aqueous concentrations calculated on the basis of addition of a known mass of neat compound and accounting for partitioning into the headspace of the bottles. Subsequently, a second experiment was set up identically, except that both benzene and toluene (90 μM each) were added simultaneously to three replicates inoculated with the nitrate-reducing culture. Sterile aqueous control bottles were prepared with 100 mL of defined mineral medium in 250-mL glass bottles capped with Mininert valves and amended with 300 μM each of benzene and toluene. All culture bottles were incubated statically in the dark in an anaerobic chamber (Coy Laboratories Products Inc., Ann Arbor, MI) filled with a gas mixture containing 80% N_2, 10% H_2, and 10% CO_2.

Labeled ^{13}C-Benzene Experiments. These experiments were conducted in 1-L enrichment cultures that were grown in 1150-mL glass bottles (VWR), with tops modified by a glassblower to accept screwcap Mininert valves (Supelco). Prior to adding ^{13}C-labeled benzene to these cultures, the concentrations of residual benzene and toluene were verified to be below detection (<11 nM and <4.3 nM, respectively). Two 1-L nitrate-reducing (NR) cultures were amended with 240 μM $^{13}C_6$-benzene. Similarly, two 1-L methanogenic (M) cultures were amended with 430 μM $^{13}C_6$-benzene. One or two days later, unlabeled benzoate (110 μM or 290 μM in the NR and M cultures, respectively) was added to one of the two replicate cultures to trap potential labeled metabolites. The concentration of benzoate added was approximately 10 times that tentatively detected in extracts from the culture in previous experiments (data not shown). Previous studies have also shown that these concentrations inhibited benzene mineralization (*11*). Cultures (1 L) amended with unlabeled benzene with and without benzoate were also similarly prepared to test the effects of benzoate on benzene degradation. Benzene, toluene, and methane were measured in headspace samples of these cultures over the course of degradation. Nitrate and nitrite were measured in 1-mL liquid samples from the nitrate-reducing cultures. In addition, liquid samples (100 mL) were periodically removed, extracted, and concentrated for GC/MS analysis for potential metabolites, as described below.

Experimental Controls. To control for leaks, abiotic transformations, and feedstock purity, four control bottles containing anaerobic mineral medium and $^{13}C_6$-benzene were also prepared. Control bottles were sampled and analyzed at the same time and in the same manner as the cultures. This analysis revealed that purchased $^{13}C_6$-benzene (99.9% purity) was contaminated with 0.05% (mol/mol) toluene. The 0.05% toluene contamination was found to consist of about 78% unlabeled $^{12}C_7$-toluene, 17% universally labeled $^{13}C_7$-toluene, and 5% ring-labeled $^{13}C_6$-toluene (see Results and Discussion). Time-zero (i.e., immediately after adding $^{13}C_6$-benzene) concentrations of the different species of toluene in active cultures were calculated on the basis of the GC/PID-measured concentration (see Analytical Procedures) of toluene and the 78% ($^{12}C_7$-), 17% ($^{13}C_7$-), and 5% ($^{13}C_6$-) breakdown of contaminating toluene. This calculation was necessary because degradation had already begun by the time the initial GC/MS samples (see below) were taken 1−2 h later. Phenol, benzoate, and unlabeled benzene were never detected in the sterile control bottles.

Extraction Procedure. The procedure described by Beller et al. (*35*) was used to extract culture liquid. Four samples were taken over the course of degradation. Samples (100 mL) were withdrawn using a glass syringe while inside an anaerobic glovebox and were placed in acid-washed glassware and were capped with Mininert valves. Ethylbenzene from an aqueous stock solution was added to the culture samples to an aqueous concentration of 900 nM as an internal standard. The samples were acidified to pH ≤ 2 with HCl, followed by the addition of 50 mL of diethyl ether (high purity, distilled-in-glass; J. T. Baker). The samples were acidified to protonate organic acids such as benzoate, thereby making them more soluble in the diethyl ether. The samples were extracted three times with 50 mL of diethyl ether. The three extracts were combined and the combined sample (150 mL) was dried over anhydrous sodium sulfate. The solutions were then concentrated under a gentle stream of high-purity nitrogen on ice to a final volume of about 100 μL of ether. Just prior to analysis, the volume of the ether extracts was measured and adjusted to exactly 100 μL, if necessary. Samples of this concentrated ether extract were analyzed by gas chromatography/mass spectrometry (GC/MS) as described below.

Analytical Procedures. Benzene, toluene, and methane concentrations were measured by removing a 300-μL headspace sample from a culture or control bottle with a 500-μL Pressure-Lok gas syringe (Precision Sampling Corp., Baton Rouge, LA) and injecting the sample into a Hewlett-Packard 5890 Series II GC equipped with a J&W Scientific GSQ 30-m

TABLE 1. Labeled and Unlabeled Toluene and Benzoate Aqueous Concentrations during $^{13}C_6$-Benzene Degradation in the Nitrate-Reducing Enrichment Culture

	GC headspace analysis[a]		GC/MS liquid analysis (nM)			
time (days)	benzene (μM) (FID)	toluene (nM) (PID)	$^{13}C_6$-toluene (ring-labeled)	$^{13}C_7$-toluene (universally labeled)	$^{12}C_7$-toluene (unlabeled)	$^{13}C_6$-benzoate (ring-labeled)
With Benzoate Trap (Added on Day 2)						
0[b]	220	200	9.5	33	150	<0.47
0.06	220	190	43	22	95	12
11	160	94	85	2.1	5.5	25
23	53	62	51	4.8	3.4	116
32	<11	<4.3	12	1.3	3.5	32
Without Benzoate Trap						
0[b]	210	160	7.4	26	120	<0.47
1	190	160	76	9.0	54	0.65
3	110	190	140	9.9	18	2.0
5	53	120	83	1.3	29	2.1
11	11	<4.3	1.2	3.5	1.2	1.8
Detection Limits for Each Analysis						
	11	4.3	0.51	0.51	0.54	0.47

[a] Benzene was detected with a flame ionization detector (FID), while toluene was detected with a much more sensitive photoionization detector (PID). [b] Time-zero GC/MS data were calculated using the GC/PID-measured concentration of toluene in the culture and the 78% ($^{12}C_7$-toluene), 17% ($^{13}C_7$-toluene), and 5% ($^{13}C_6$-toluene) breakdown found in the feedstock (see Table 2; footnote c). This calculation was necessary since degradation had already begun by the time the first GC/MS samples were taken. All benzene detected was ^{13}C-labeled.

TABLE 2. Labeled and Unlabeled Toluene, Phenol, and Benzoate Aqueous Concentrations during $^{13}C_6$-Benzene Degradation in the Methanogenic Enrichment Culture

	GC headspace analysis[a]		GC/MS liquid analysis (nM)					
time (days)	benzene (μM) (FID)	toluene (nM) (PID)	$^{13}C_6$-toluene (ring-labeled)	$^{13}C_7$-toluene (universally labeled)	$^{12}C_7$-toluene (unlabeled)	$^{13}C_6$-phenol (ring-labeled)	$^{12}C_6$-phenol (unlabeled)	$^{13}C_6$-benzoate (ring-labeled)
With Benzoate Trap (Added on Day 1)								
0[b]	420	170	7.9	28	130	<1.0	<1.1	<0.47
0.06	410	160	36	23	38	50	7.4	31
5	200	120	42	23	17	6900	1.1	3300
11	55	70	46	5.3	56	38	3.2	3000
22	<11	<4.3	0.47	3.6	61	<1.0	<1.1	3900
Without Benzoate Trap								
0[b]	330	110	5.2	18	85	<1.0	<1.1	<0.47
0.08	330	110	29	15	12	5.5	<1.1	59
2	225	94	14	5.6	6.3	1.7	<1.1	68
9	95	87	1.8	1.7	1.3	1.0	<1.1	25
18	<11	<4.3	<0.51	<0.51	<0.54	2.8	<1.1	<0.47
Sterile Controls								
0	240	110	0.71	21	58	<1.0	<1.1	<0.47
11	250	130	5.7	15	86	<1.0	<1.1	<0.47
23	250	97	6.8	12	89	<1.0	<1.1	<0.47
32	250	82	2.1	6.5	25	<1.0	<1.1	<0.47
average[c]			3.8	14	65	<1.0	<1.1	<0.47
Detection limits for each analysis								
	11	4.3	0.51	0.51	0.54	1.0	1.1	0.47

[a] Benzene was detected with a flame ionization detector (FID), while toluene was detected with a much more sensitive photoionization detector (PID). [b] Time-zero GC/MS data were calculated using the GC/PID-measured concentration of toluene in the culture and the 78% ($^{12}C_7$-toluene), 17% ($^{13}C_7$-toluene), and 5% ($^{13}C_6$-toluene) breakdown found in the feedstock (see footnote c). This calculation was necessary since degradation had already begun by the time the first GC/MS samples were taken. All benzene detected was ^{13}C-labeled. [c] The purchased $^{13}C_6$-benzene (99.9% purity) was contaminated with 0.05% (mol/mol) toluene. The 0.05% toluene contamination consisted of about 78% unlabeled $^{12}C_7$-toluene, 17% universally labeled $^{13}C_7$-toluene, and 5% ring-labeled $^{13}C_6$-toluene. For example, the mole percent $^{13}C_6$-toluene in the benzene feedstock was calculated from the average measured in sterile bottles as follows: [(3.8 nM)/(3.8 nM + 14 nM + 65 nM)] × 100 = 5%.

by 0.53-mm (inner diameter) PLOT column and a photoionization detector (PID) and flame ionization detector (FID) in series. The injector temperature was 200 ℃, the oven temperature was held isothermally at 190 ℃, and the detector temperature was 250 ℃. The carrier gas was helium at a flow rate of 11 mL/min. Calibration was with external standards, the error for benzene and toluene concentration measurements was ±5%, and the benzene and toluene detection limits were 12 μM and 10 μM, respectively, with the flame ionization detector. Toluene concentrations were also measured with a more sensitive photoionization detector (PID) that was situated in-line in the GC before the FID. The toluene detection limit was 5 nM with the PID. The analytical error for toluene concentrations was determined with duplicate sterile standard bottles containing 200, 110, and 50 nM of toluene. The standard bottles were sampled every time an experimental sample was taken (n = 20). The standard deviations in these controls were 26, 24, and 20 nM for the three concentrations of toluene, respectively. Nitrate and nitrite concentrations were analyzed by autosampler injection of 20 μL of diluted liquid samples onto a Dionex 300 Series ion chromatograph with an IonPac AS14 4-mm column. The

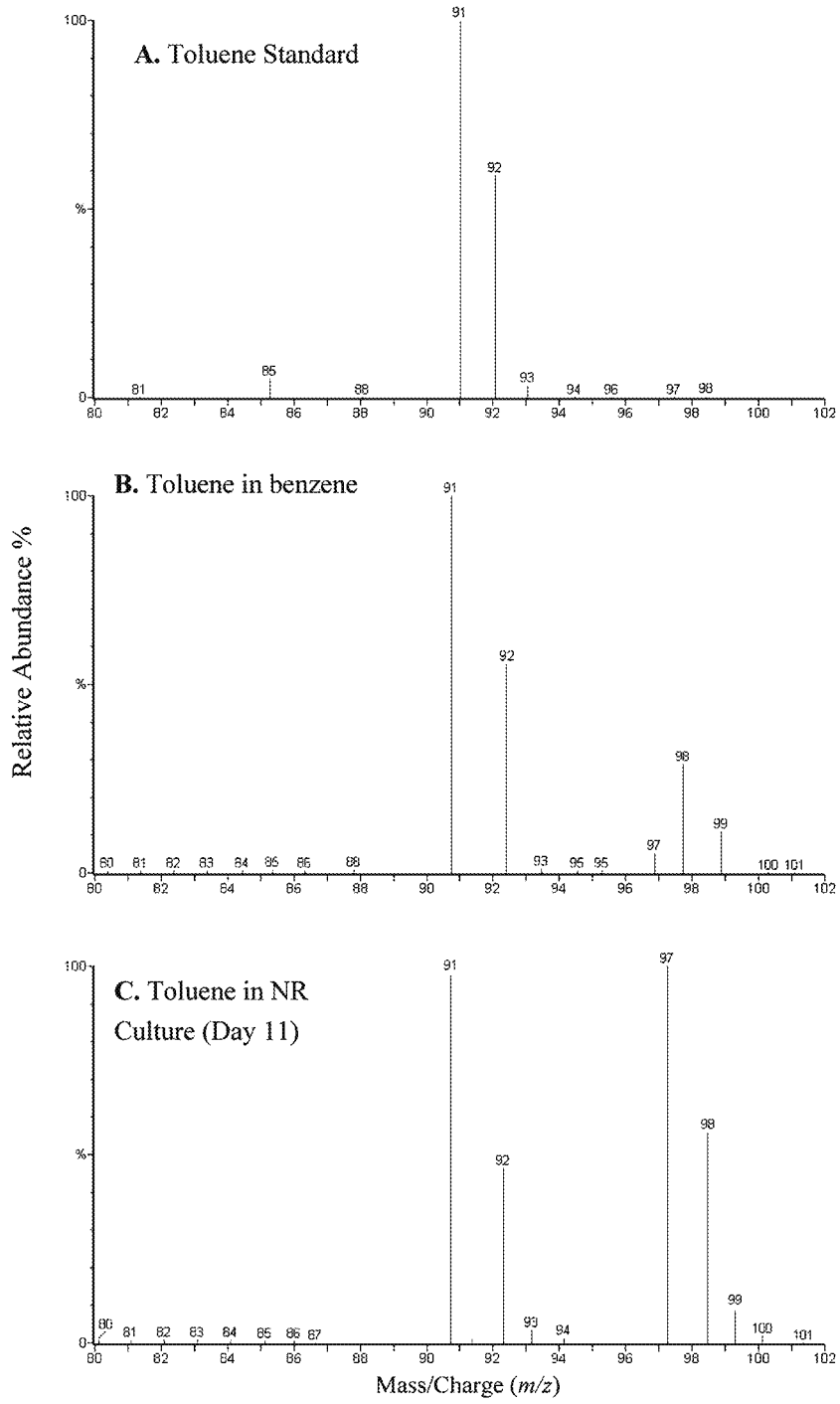

FIGURE 1. Mass spectra at the retention time (4.6 min) of toluene. (A) Mass spectrum of unlabeled toluene standard. The fragments at *m/z* 91, 92, and 93 occur at relative abundances of roughly 100 (base peak), 55, and 5%, respectively. (B) Mass spectrum of toluene contamination found in the neat $^{13}C_6$-benzene feeding stock (and therefore present at time zero). The toluene contamination consisted of 78% unlabeled $^{12}C_7$-toluene (base peak at *m/z* 91), 17% universally labeled $^{13}C_7$-toluene (base peak at *m/z* 98), and 5% ring-labeled $^{13}C_6$-toluene (base peak at *m/z* 97). (C) Mass spectrum of toluene detected in the nitrate-reducing (NR) culture with a benzoate trap on day 24. Similar spectra were found in the experiment without benzoate.

eluent consisted of 3.5 mM Na_2CO_3 and 1 mM $NaHCO_3$ and was run isocratically at a flow rate of 1.2 mL/min. The errors for nitrite and nitrate concentrations were ±0.5 and ±2.4%, respectively. The detection limit for the anions was 0.02 mM.

GC/MS Analysis. To identify metabolites of benzene degradation, 5 μL of the 100-μL ether extract (prepared as described above) was injected into a Perkin-Elmer TurboMass with Autosystem XL GC/MS system equipped with TurboMass Software Version 2.0 and a ZB-WAX fused silica capillary column (30-m length, 0.35-mm inner diameter, 0.25-μm film thickness, Phenomenex, Torrance, CA). The retention times of benzene, toluene, ethylbenzene, methyl benzoate, phenol, and benzoate were 3.4, 4.6, 6.4, 11.2, 13.5, and 16.5 min, respectively. The injector temperature was 250 °C and the oven temperature was held at 25 °C using dry ice for 5 min and then was raised at a rate of 10 °C/min to 200 °C for 10

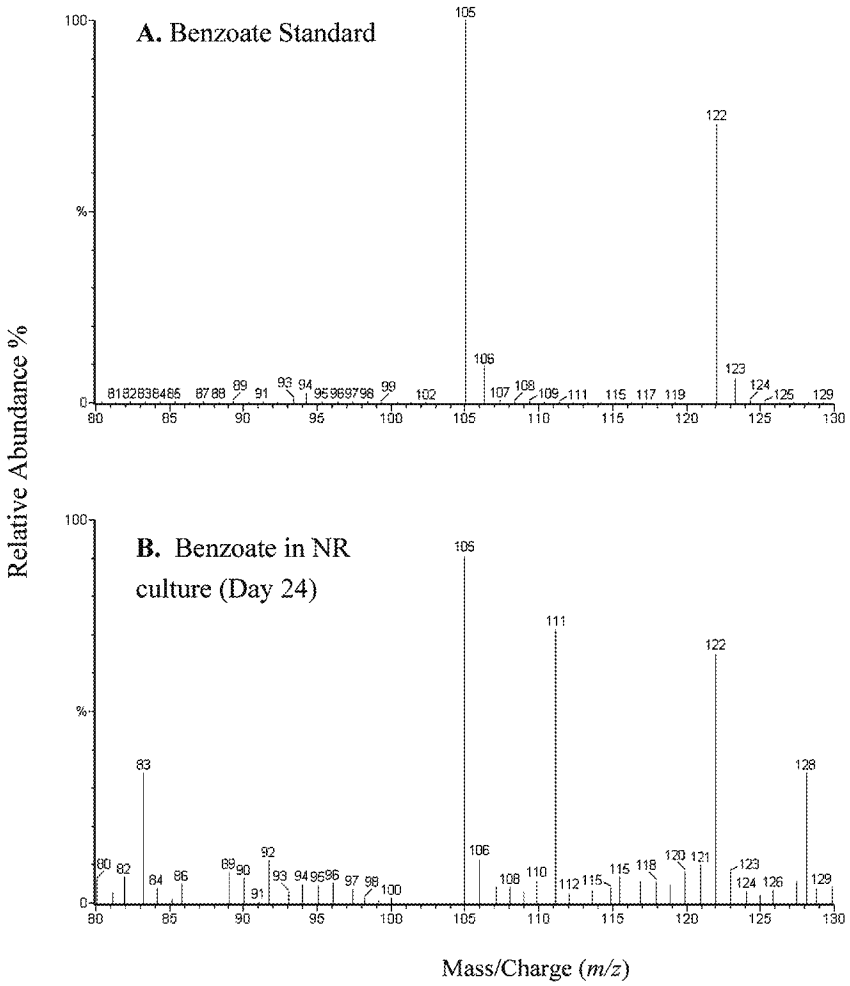

FIGURE 2. Mass spectra at the retention time (16.3 min) of benzoate. (A) Mass spectrum of unlabeled benzoate standard. The fragments at *m/z* 105, 106, 122, and 123 occur at relative abundances of roughly 100 (base peak), 10, 80, and 9%, respectively. (B) Mass spectrum of benzoate detected in the nitrate-reducing (NR) culture with a benzoate trap on day 24.

min. The split was opened at 0.5 min with a flow of 15 mL/min. Each sample was analyzed in both full scan mode and selected ion mode.

GC/MS Calibration. Calibration curves were constructed using unlabeled external standards made in ether and analyzed in selected ion mode on the GC/MS. The ion fragment corresponding to the base peak was used for quantification. For unlabeled toluene, the area of the peak obtained using only data for the ion corresponding to the base peak in the spectrum at mass-to-charge ratio (m/z) of 91 was used to construct the calibration curves. To calculate $^{13}C_6$-toluene concentrations, the data for the ion at m/z 97 was used. Using this selected ion mode, the detection limits for $^{13}C_6$-toluene, $^{13}C_6$-benzoate, and $^{13}C_6$-phenol were 0.51, 0.47, and 1.0 nM, respectively. Concentrations of phenol, toluene, ethylbenzene, and benzoate in ether extracts were determined from external calibration curves of standards in ether and then related back to aqueous concentrations in the original culture assuming 100% extraction efficiency (100 mL culture concentrated to 100 μL ether; 5 μL ether injected). This results in a conservative underestimation of the concentrations in the culture. Since differences in purging between samples could significantly affect the recovery of volatile constituents such as toluene, the area of the internal standard peak, ethylbenzene, was checked for consistency between samples. The recovery of ethylbenzene was relatively similar between samples (average 60 ± 25%). Therefore, no adjustments were made for differences in ethylbenzene recoveries between samples.

Results and Discussion

Toluene and Benzoate Degradation Experiments. These experiments were carried out to determine if cultures maintained solely on benzene could also degrade the putative metabolites toluene and benzoate. The nitrate-reducing cultures degraded toluene rapidly, more than 2.5 times faster than benzene with identical inocula (data not shown). When toluene and benzene were amended simultaneously, toluene was degraded first, while benzene degradation began 10 days later, once toluene was depleted (data not shown). Benzene degradation in the NR cultures was also inhibited in the presence of benzoate (data not shown). Benzoate concentrations were not directly measured but could be inferred from nitrate depletion data. Once benzoate was depleted, benzene degradation resumed. Toluene degradation was also inhibited by benzoate in these cultures (data not shown). In contrast, benzene degradation in the absence of toluene or benzoate proceeded without delay (data not shown).

In contrast to the rapid toluene degradation observed in the nitrate-reducing cultures, toluene at a concentration of 110 μM was not appreciably degraded by the methanogenic benzene-degrading culture (data not shown). When benzene and toluene were amended simultaneously at similar concentrations, benzene degradation proceeded uninhibited,

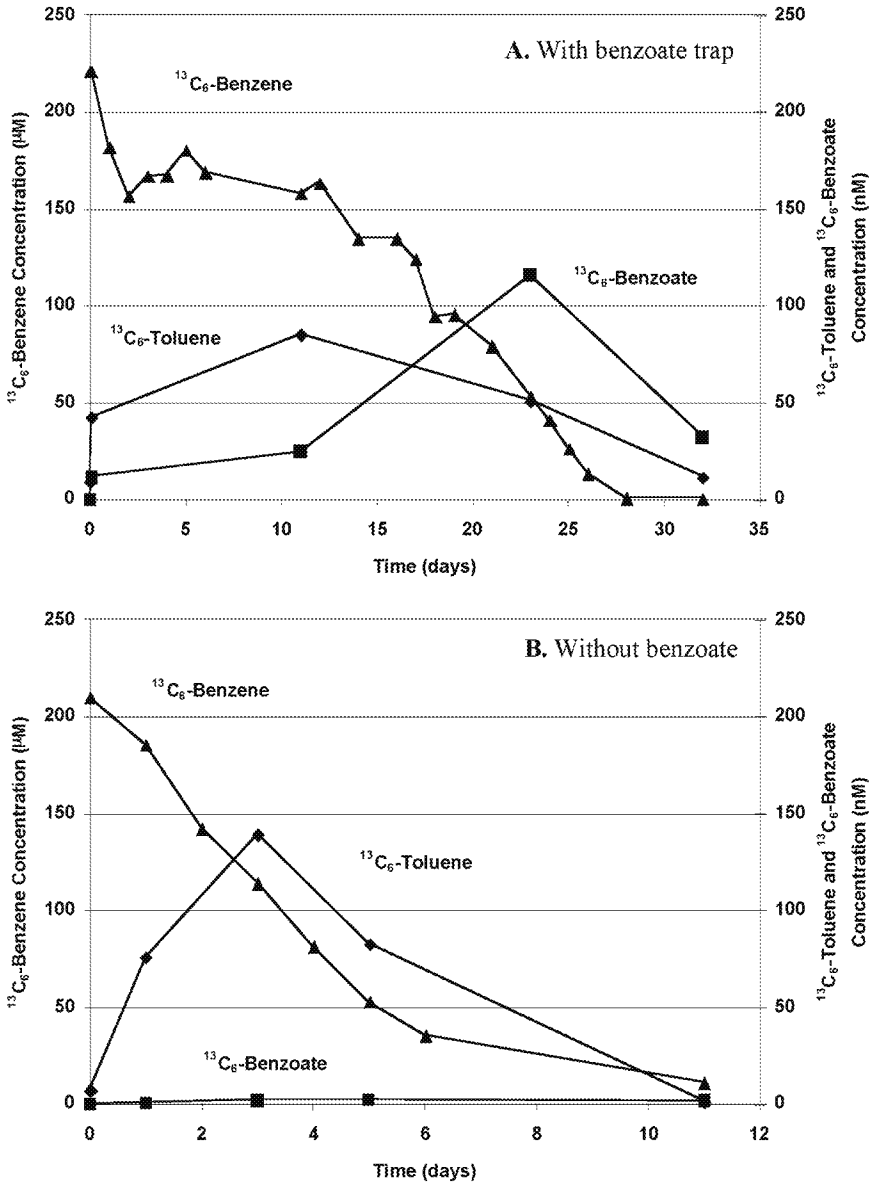

FIGURE 3. Concentrations of labeled metabolites over time in the nitrate-reducing culture. Concentration of $^{13}C_6$-toluene (diamonds) and $^{13}C_6$-benzoate (squares) during $^{13}C_6$-benzene (triangles) degradation with a benzoate trap (A) and without benzoate (B).

while less than 10% of the toluene was degraded. Similarly, benzene degradation in the methanogenic cultures was not markedly inhibited by the presence of benzoate (data not shown). Only 20% of the benzoate added was degraded. Moreover, the rate of benzene degradation was not affected by the presence of toluene or benzoate. While the lack of degradation of benzoate at these concentrations could be the result of inefficient uptake into the cell or inability to form the CoA thioester (*36*), these explanations cannot be invoked for toluene, which can readily pass through the membrane. These results will be revisited, after the metabolite experiments are described.

$^{13}C_6$-Benzene Experiments. During benzene biodegradation, 100-mL samples from culture bottles and 100-mL samples from control bottles were removed at the times indicated in Tables 1 and 2, were extracted with ether, and the extracts were analyzed by GC/MS. In the control bottles, this analysis revealed that the purchased $^{13}C_6$-benzene stock contained only $^{13}C_6$-benzene (i.e., no unlabeled $^{12}C_6$-benzene) but was contaminated with toluene. Fortunately, however, the concentration of contaminating toluene, particularly $^{13}C_6$-toluene, was significantly lower than concentrations ultimately measured in the cultures and thus did not affect interpretation of the results. GC/MS analysis of samples from cultures revealed the presence of compounds that eluted at the retention times of toluene, benzoate, and phenol (the latter only in the methanogenic cultures). If toluene, benzoate, and phenol were transformation products of $^{13}C_6$-benzene, then key mass spectral fragments of these compounds should exhibit a shift of +6 mass units relative to the unlabeled compound. The GC/MS method and column used in these experiments could also resolve other potential metabolites, including benzaldehyde and benzyl alcohol. None of these compounds were detected in culture samples. The method was not able to detect 4-hydroxybenzoate.

Metabolites Detected in the Nitrate-Reducing Culture: A. Toluene. The mass spectrum of an authentic toluene standard consists primarily of fragments at m/z 91, 92, and 93 with relative abundances of 100, 55, and 5%, respectively (Figure 1A). The mass spectrum of toluene found to contaminate the purchased $^{13}C_6$-benzene (Figure 1B) and the mass spectra of toluene in culture extracts (Figure 1C) included fragments corresponding to a mixture of unlabeled ($^{12}C_7$), ring-labeled ($^{13}C_6$), and universally labeled ($^{13}C_7$) toluene. Mass spectral features that were +6 mass units relative to the toluene standard (with the same relative

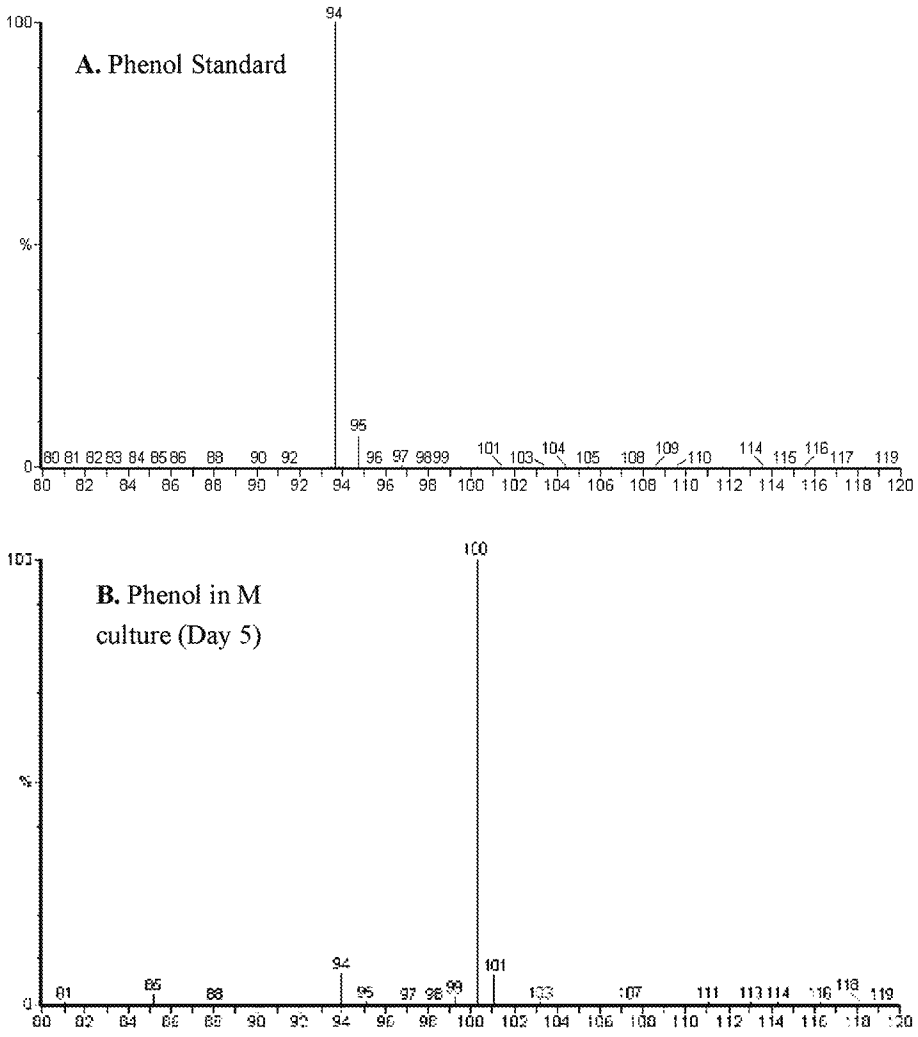

FIGURE 4. Mass spectra at the retention time (13.5 min) of phenol. (A) Mass spectrum of unlabeled phenol standard. The fragments at *m/z* 94 and 95 occur at relative abundances of roughly 100 (base peak) and 6%, respectively. (B) Mass spectrum of phenol detected in the methanogenic (M) culture with a benzoate trap on day 5. Similar spectra were found in the experiment without benzoate.

abundance), that is, fragments at m/z 97, 98, and 99, correspond to $^{13}C_6$-toluene. Mass spectral features that were +7 mass units relative to the toluene standard (i.e., m/z of 98, 99, and 100) correspond to $^{13}C_7$-toluene. The concentration of each different labeled toluene species in each sample was determined from the area of the ion chromatogram for the base peak (m/z 91, 97, or 98), taking into consideration that the area of the peak for m/z 98 includes contributions from both $^{13}C_6$-toluene (55% of the area determined using m/z 97) and $^{13}C_7$-toluene. These data were tabulated for cultures (Tables 1) and controls (Table 2) over time and were compared to total toluene concentrations measured by headspace analysis on a PID (Table 1) for confirmation. Good agreement was obtained between the sum of the concentration of the three toluene species determined by GC/MS and the concentration of total toluene measured by GC/PID (Table 1). $^{13}C_6$-toluene concentrations increased markedly (>10 times) above the amount introduced as contamination from the $^{13}C_6$-benzene feeding stock (Table 1). In contrast, none of the other contaminating toluene species (unlabeled and $^{13}C_7$-toluene) increased in concentration during these experiments. These data conclusively identify toluene as a metabolite of benzene in these cultures.

Metabolites Detected in the Nitrate-Reducing Culture: B. Benzoate. The mass spectrum of an authentic unlabeled benzoate standard consists primarily of fragments at m/z 105, 106, 122, and 123 (Figure 2A). No $^{13}C_6$- or $^{13}C_7$-benzoate was detected in the controls (Table 2) or in the nitrate-reducing enrichment culture amended with unlabeled benzene (data not shown). During degradation of $^{13}C_6$-benzene, a peak at the retention time of benzoate (RT = 16.3 min) was observed. The mass spectral profile at this retention time indicated a mixture of $^{12}C_7$ (unlabeled) and $^{13}C_6$-labeled benzoate but no $^{13}C_7$-benzoate (Figure 2B). The mass spectral features observed were +6 mass units relative to a benzoate standard: fragments at m/z 105 and 122 in unlabeled benzoate were observed at m/z 111 and 128, respectively, in the culture extract (Figure 2B). Aqueous concentrations of $^{13}C_6$-benzoate in all culture samples extracted were determined from the areas of the base peak, as was done for toluene. Labeled benzoate concentrations increased notably during benzene degradation, especially in the cultures with the added unlabeled benzoate trap (Table 1 and Figure 3). These data confirm that benzoate is an intermediate of anaerobic benzene degradation in these cultures.

Metabolites Detected in the Methanogenic Culture: A. Phenol. The mass spectrum of an authentic phenol standard consists primarily of fragments at m/z 94 and 95 (Figure 4A). The mass spectrum at the retention time of phenol for samples from the culture included fragments that were shifted by +6 mass units relative to a phenol standard (Figure 4B). In the samples from the culture with benzoate added, the spectrum indicated a mixture of $^{12}C_6$ (unlabeled) and $^{13}C_6$-labeled phenol (Figure 4B). In samples from the culture

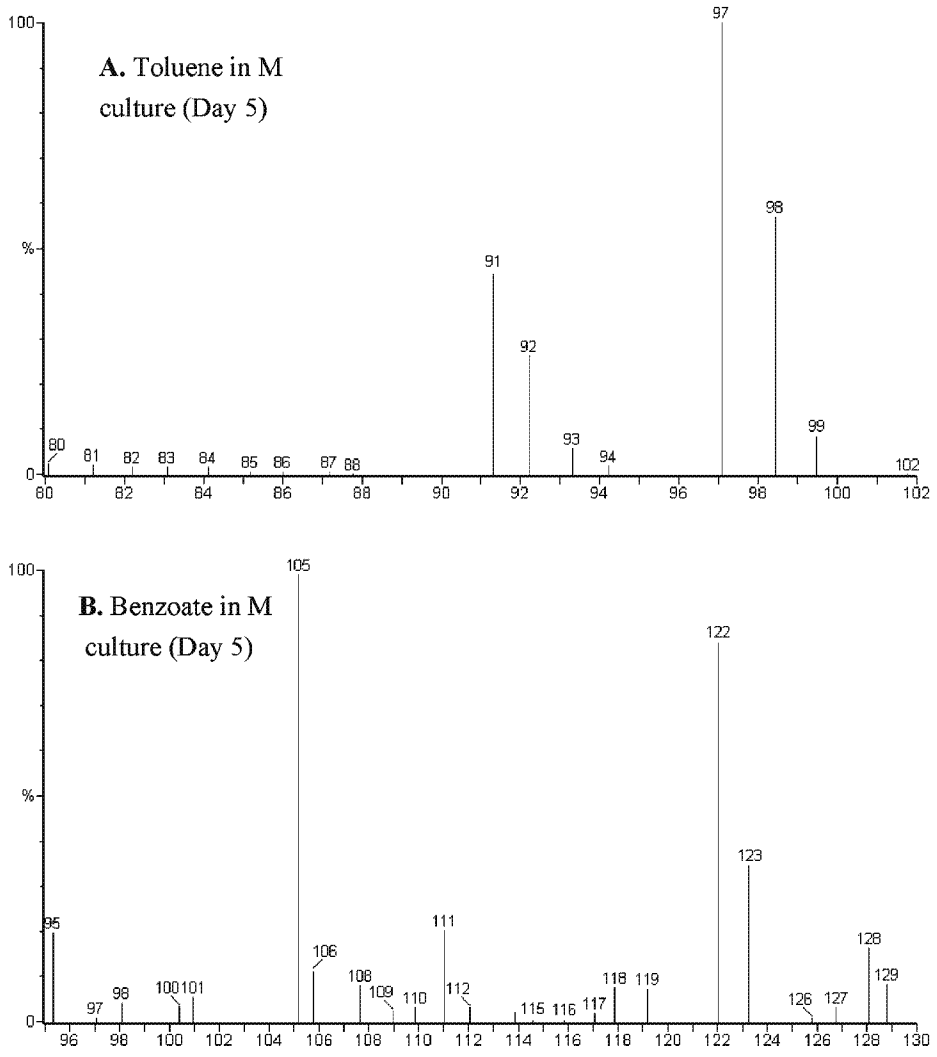

FIGURE 5. Mass spectra from methanogenic culture. (A) Mass spectrum of toluene (ret. time 4.6 min) and (B) mass spectrum of benzoate (ret. time 16.3 min) detected in the methanogenic (M) culture with a benzoate trap on day 5.

without benzoate, only $^{13}C_6$-labeled phenol was detected (Table 2). These data confirm that phenol was a product of benzene transformation in these cultures. The fact that unlabeled phenol was detected only in the culture amended with high concentrations of unlabeled benzoate may indicate that this phenol was produced from the benzoate in a reverse reaction from those described for phenol transformation to benzoate (*29*, *40*).

Metabolites Detected in the Methanogenic Culture: B. Toluene. The mass spectrum at the retention time of toluene for samples from the methanogenic culture indicated a mixture of unlabeled, ring-labeled, and universally labeled toluene (Figure 5A). These mass spectra were similar to those found for the nitrate-reducing enrichment cultures, however, the concentrations of ring-labeled toluene were slightly lower than in the nitrate-reducing culture (Table 2). As with the nitrate-reducing cultures, only the concentration of ring-labeled toluene increased during degradation; universally labeled and unlabeled toluene concentrations decreased continuously from initial concentrations. These data strongly implicate toluene as a product of benzene transformation in these cultures.

Metabolites Detected in the Methanogenic Culture: C. Benzoate. The mass spectrum at the retention time of benzoate for samples from the methanogenic culture was similar to those observed in the nitrate-reducing culture (Figure 5B). Concentrations of ring-labeled benzoate increased considerably, especially in the cultures with a benzoate trap (Table 2), confirming that benzoate was a metabolite of benzene in these cultures.

Time Course of Metabolite Formation and Implications for Reaction Pathways. To clearly visualize the relationship between $^{13}C_6$-benzene degradation and the appearance of $^{13}C_6$-labeled metabolites, the molar concentrations of these compounds were plotted as a function of time for the nitrate-reducing cultures (Figure 3A and 3B) and methanogenic cultures (Figure 6A and 6B). In the nitrate-reducing cultures, toluene concentrations first increased, then decreased, as one would expect for a metabolic intermediate. In the culture with the benzoate trap (Figure 3A), the labeled benzoate concentration reached a higher maximum, and toluene formation and subsequent degradation clearly preceded that of benzoate, which is consistent with toluene conversion to benzoate. Note that a rapid increase in $^{13}C_6$-toluene was observed in the first few hours of degradation (see Table 1) that is difficult to see on the time scale shown in Figure 3A. As with toluene, benzoate concentrations increased first, prior to decreasing over the course of benzene degradation. These data are entirely consistent with some form of benzene methylation reaction preceding the transformation of toluene to benzoate. Benzylsuccinate, an intermediate in the anaerobic transformation of toluene to benzoate (or its CoA thioester), could not be detected with the analysis methods used. Confirmation of benzylsuccinate formation was sought

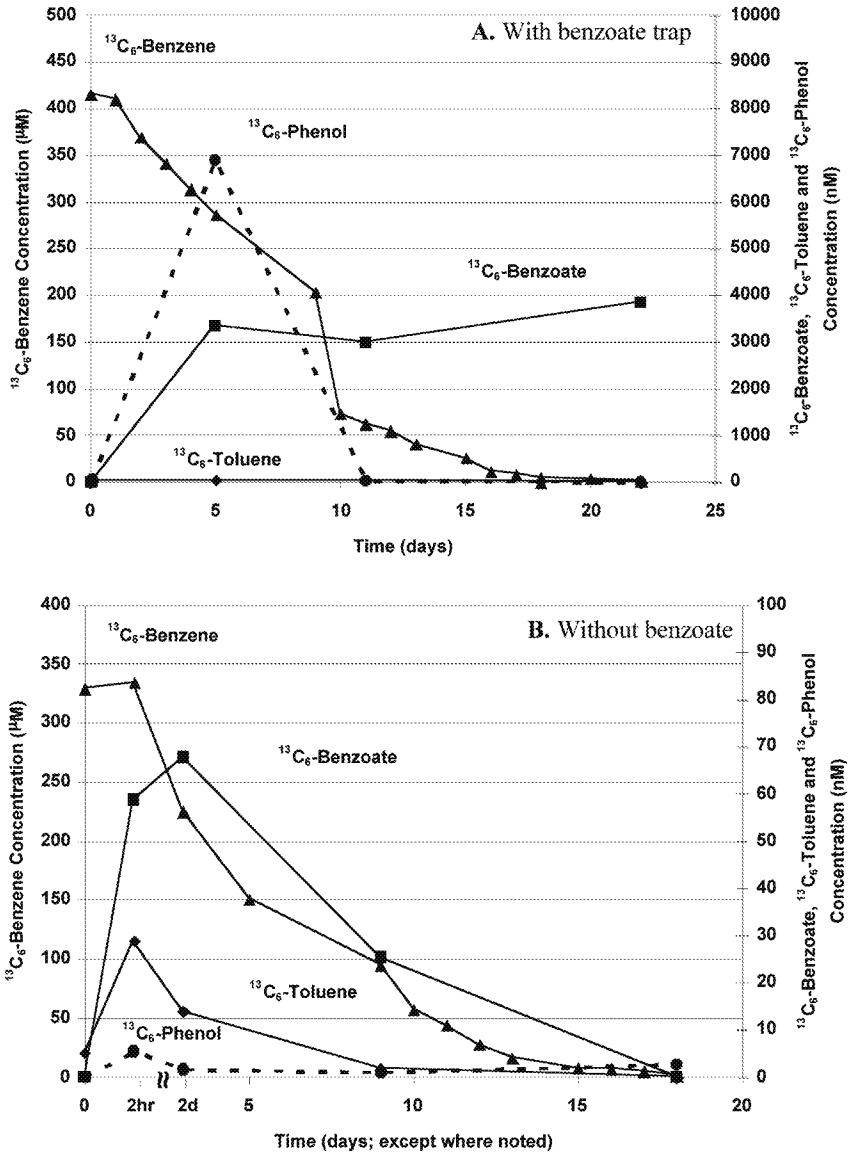

FIGURE 6. Concentrations of labeled metabolites over time in the methanogenic culture. Concentration of $^{13}C_6$-toluene (diamonds), $^{13}C_6$-phenol (circles), and $^{13}C_6$-benzoate (squares) during $^{13}C_6$-benzene (triangles) degradation in the methanogenic culture with a benzoate trap (A) and without benzoate (B).

by liquid chromatography/tandem mass spectrometry analysis of ether extracts (*37*), but none could be detected. It is possible that the concentration of benzylsuccinate was simply too low to be detected, given the relatively low accumulated toluene concentrations; however, this still needs to be verified.

In the methanogenic cultures, labeled phenol, as well as labeled toluene and benzoate, was detected. Phenol concentrations initially increased, most significantly in the culture with the benzoate trap (Figure 6A), and subsequently decreased, consistent with phenol being an intermediate in the degradation pathway. $^{13}C_6$-phenol and $^{13}C_6$-benzoate concentrations increased to 7 μM and 4 μM, respectively, the highest concentrations of these metabolites ever detected, representing over 3 mol % of the benzene degraded at that time (~150 μM). Interestingly, the relatively high concentrations of benzoate that accumulated in the culture did not subsequently decrease as benzene became depleted (Figure 6A). It could be that this represented benzoate that had somehow been released from the cell and could not be metabolized further; this is consistent with the earlier observation that the culture does not metabolize benzoate readily. Phenol and benzoate were also transiently detected in the culture without benzoate added (Figure 6B) but at much lower concentrations (50- to 1000-fold lower).

Ring-labeled $^{13}C_6$-toluene was also transiently detected in these methanogenic cultures. The concentrations of $^{13}C_6$-toluene detected in these cultures were somewhat lower than those in the nitrate-reducing cultures but were still well above the concentrations introduced at time zero and therefore are clear evidence that benzene may have been methylated in these methanogenic cultures, either in parallel or in series with the formation of phenol and benzoate. Since the methanogenic culture comprises many different species, it is possible that several pathways were operative simultaneously in different organisms.

The pathway for anaerobic benzene biodegradation has eluded researchers for the past decade. Benzene-degrading cultures generally have long doubling times and relatively slow degradation rates and tolerate only low concentrations of benzene. Moreover, metabolite concentrations are very low because the rate-limiting step is likely the initial attack on benzene, and thus downstream metabolites barely accumulate. It has taken several years to develop highly

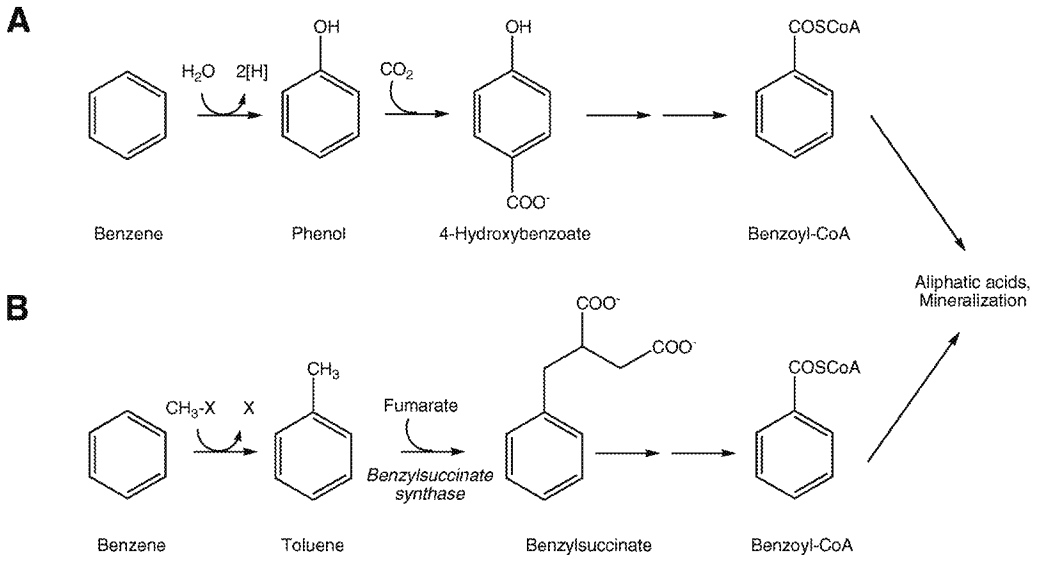

FIGURE 7. Proposed initial steps in anaerobic benzene biodegradation. (A) Benzene hydroxylation (*30, 31*). (B) Benzene methylation (*32*). In Figure 7B, X represents a methyl group carrier such as tetrahydrofolate, *S*-adenosylmethionine, or cobalamin protein.

enriched cultures in sufficient volumes such that 100 mL of culture could be sacrificed and extracted at each time point to obtain the sensitivity required for detection.

In this study, using $^{13}C_6$-benzene, we conclusively identified and (for the first time) quantified $^{13}C_6$-toluene, $^{13}C_6$-benzoate, and $^{13}C_6$-phenol over the course of benzene degradation in nitrate-reducing and methanogenic enrichment cultures. Further work is required to determine the exact mechanisms for these transformations and the nature of the alkylating and hydroxylating agents. Moreover, it would be interesting to determine if toluene is further transformed via the well-established benzylsuccinate synthase (BSS) pathway involving toluene addition to fumarate to form benzylsuccinate (Figure 7) (*25, 26*). Coates et al. (*32*) first proposed that benzene could be alkylated to toluene on the basis of preliminary observations with the nitrate-reducing, benzene-degrading isolate *Dechloromonas* strain RCB. Benzene degradation by *Dechloromonas* strain RCB was inhibited by toluene, benzoate, benzaldehyde, benzyl alcohol, or acetate, suggesting that these compounds may be potential intermediates (*32*). Furthermore, benzene degradation by this organism was stimulated by the addition of vitamin B_{12} and was inhibited by propyl iodide, a specific inhibitor of cobalamin-mediated methylation reactions (*38, 39*). Biologically mediated alkylation of benzene to toluene has previously been observed with human bone marrow incubated with *S*-adenosyl-L-methionine (*40*). A similar mechanism may be operative in these cultures. *S*-Adenosylmethionine may also be involved in the formation of a glycyl radical in BSS catalyzed by BSS-activating enzyme (*25*).

In the methanogenic enrichment cultures, we identified $^{13}C_6$-phenol, in addition to $^{13}C_6$-benzoate and $^{13}C_6$-toluene, during $^{13}C_6$-benzene degradation. Two pathways are hypothesized on the basis of our observations: (1) benzene hydroxylation to form phenol and (2) benzene methylation to form toluene (Figure 7). Evidence for hydroxylation of the benzene ring to form phenol has been noted in three previous studies. Using GC/MS, Vogel and Grbić-Galić observed the oxidation of benzene to phenol in a methanogenic culture (*10, 30*). The source of the phenol oxygen was confirmed through the use of $H_2{}^{18}O$. Further transformation of phenol was speculated to occur via ring reduction to form cyclohexanone, followed by ring cleavage to form aliphatic acids and eventually carbon dioxide and methane (*10, 30*). Weiner and Lovley (*11*) observed radioactive signals in the phenol fraction collected from samples of methanogenic enrichment cultures. Caldwell and Suflita observed less than 1 μM of $^{13}C_6$-phenol and a maximum of 4 μM ^{13}C-benzoate in cultures under methanogenic and sulfate- and Fe(III)-reducing conditions (*31*). None of the above studies reported a time course.

Several anaerobic cultures degrade phenol via an initial carboxylation step to yield 4-hydroxybenzoate followed by dehydroxylation to form benzoyl-CoA (*29, 41*) (Figure 7). The carboxylation of phenol has been observed under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions (*42*–*47*). Unfortunately, our method could not detect 4-hydroxybenzoate, and thus more work remains to confirm this pathway.

In summary, quantitative analysis of labeled metabolites demonstrated that benzene is converted to toluene and subsequently to benzoate under anaerobic conditions. These were the only metabolites detected in the nitrate-reducing cultures. Benzene appeared to also be converted to phenol in the methanogenic culture, where two parallel pathways may be operative.

Acknowledgments

We acknowledge the work of previous students toward enriching the cultures. Initial microcosm work was conducted by Marit Nales and initial enrichments were created by Siobhan Burland. We gratefully acknowledge Dan Mathers and Ying Lei of ANALEST (University of Toronto) for their help with GC/MS analysis. This research was supported by the Natural Science and Engineering Research Council of Canada through an operating grant awarded to Elizabeth Edwards and a postgraduate scholarship to Ania Ulrich.

Literature Cited

(1) Harwood, C. S.; Gibson, J. Shedding light on anaerobic benzene ring degradation: A process unique to prokaryotes? *J. Bacteriol.* **1997**, *179*, 301–309.
(2) Dean, B. J. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mut. Res.* **1985**, *154*, 153–181.
(3) Snyder, R. Overview of the toxicology of benzene. *J. Toxicol. Environ. Health.* **2000**, *61*, 339–346.
(4) Chiang, C. Y.; Salanitro, J. P.; Chai, E. Y.; Colthart, J. D.; Klein, C. L. Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer-data analysis and computer modeling. *Ground Water* **1989**, *27*, 823–834.
(5) Davis, J. W.; Klier, N. J.; Carpenter, C. L. Natural biological attenuation of benzene in groundwater beneath a manufacturing facility. *Ground Water* **1994**, *32*, 215–226.

(6) Gieg, L. M.; Kolhatkar, R. V.; McInerney, M. J.; Tanner, R. S.; Sublette, K. L.; Suflita, J. M. Intrinsic bioremediation of petroleum hydrocarbons in a gfas condensate-contaminated aquifer. *Environ. Sci. Technol.* **1999**, *33*, 2550−2560.
(7) Gibson, D. T.; Koch, J. R.; Kallio, R. E. Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. *Biochemistry* **1968**, *7*, 2653−2662.
(8) Major, D. W.; Mayfield, C. I.; Barker, J. F. Biotransformation of benzene by denitrification in aquifer sand. *Ground Water* **1988**, *26*, 8−14.
(9) Kazumi, J.; Caldwell, M. E.; Suflita, J. M.; Lovley, D. R.; Young, L. Y. Anaerobic degradation of benzene in diverse anoxic environments. *Environ. Sci. Technol.* **1997**, *31*, 813−818.
(10) Grbić-Galić, D.; Vogel, T. M. Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* **1987**, *53*, 254−260.
(11) Weiner, J. M.; Lovley, D. R. Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. *Appl. Environ. Microbiol.* **1998**, *64*, 1937−1939.
(12) Burland, S. M.; Edwards, E. A. Anaerobic benzene biodegradation linked to nitrate reduction. *Appl. Environ. Microbiol.* **1999**, *65*, 529−533.
(13) Coates, J. D.; Chakraborty, R.; Lack, J. G.; O'Connor, S. M.; Cole, K. A.; Bender, K. S.; Achenbach, L. A. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* **2001**, *411*, 1039−1043.
(14) Lovley, D. R.; Woodward, J. C.; Chapelle, F. H. Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. *Nature* **1994**, *370*, 128−131.
(15) Lovley, D. R.; Woodward, J. C.; Chapelle, F. H. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **1996**, *62*, 288−291.
(16) Anderson, R. T.; Rooney-Varga, J. N.; Gaw, C. V.; Lovley, D. R. Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ. Sci. Technol.* **1998**, *32*, 1222−1229.
(17) Caldwell, M. E.; Tanner, R. S.; Suflita, J. M. Microbial metabolism of benzene and the oxidation of ferrous iron under anaerobic conditions: Implications for bioremediation. *Anaerobe* **1999**, *5*, 595−603.
(18) Edwards, E. A.; Grbić-Galić, D. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. *Appl. Environ. Microbiol.* **1992**, *58*, 2663−2666.
(19) Lovley, D. R.; Coates, J. D.; Woodward, J. C.; Phillips, E. J. P. Benzene oxidation coupled to sulfate reduction. *Appl. Environ. Microbiol.* **1995**, *61*, 953−958.
(20) Phelps, C. D.; Kazumi, J.; Young, L. Y. Anaerobic degradation of benzene in BTX mixtures dependent on sulfate reduction. *FEMS Microbiol. Lett.* **1996**, *145*, 433−437.
(21) Weiner, J. M.; Lovley, D. R. Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. *Appl. Environ. Microbiol.* **1998**, *64*, 775−778.
(22) Phelps, C. D.; Young, L. Y. Anaerobic biodegradation of BTEX and gasoline in various aquatic sediments. *Biodegradation* **1999**, *10*, 15−25.
(23) Reinhard, M.; Shang, S.; Kitanidis, P. K.; Orwin, E.; Hopkins, G. D.; Lebron, C. A. In situ BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. *Environ. Sci. Technol.* **1997**, *31*, 28−36.
(24) Anderson, R. T.; Lovley, D. R. Anaerobic bioremediation of benzene under sulfate reducing conditions in a petroleum contaminated aquifer. *Environ. Sci. Technol.* **2000**, *34*, 2261−2266.
(25) Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F. Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol. Rev.* **1998**, *22*, 459−473.
(26) Spormann, A. M.; Widdel, F. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **2000**, *11*, 85−105.
(27) Phelps, C. D.; Battistelli, J.; Young, L. Y. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation in situ. *Environ. Microbiol.* **2002**, *4*, 532−537.
(28) Meckenstock, R. U.; Annweiler, E.; Michaelis, W.; Richnow, H. H.; Schink, B. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* **2000**, *66*, 2743−2747.
(29) Heider, J.; Fuchs, G. Anaerobic metabolism of aromatic compounds. *Eur. J. Biochem.* **1997**, 577−596.
(30) Vogel, T. M.; Grbić-Galić, D. Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. *Appl. Environ. Microbiol.* **1986**, *52*, 200−202.
(31) Caldwell, M. E.; Suflita, J. M. Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron accepting conditions. *Environ. Sci. Technol.* **2000**, *34*, 1216−1220.
(32) Coates, J. D.; Chakraborty, R.; McInerney, M. J. Anaerobic benzene biodegradation - A new era. *Res. Microbiol.* **2002**, *153*, 621−628.
(33) Nales, M.; Butler, B.; Edwards, E. Anaerobic benzene biodegradation: a microcosm survey. *Biorem. J.* **1998**, *2*, 125−144.
(34) Ulrich, A. C.; Edwards, E. A. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. *Environ. Microbiol.* **2003**, *5*, 92−102.
(35) Beller, H. R.; Reinhard, M.; Grbić-Galić, D. Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. *Appl. Environ. Microbiol.* **1992**, *58*, 3192−3195.
(36) Harwood, C. S.; Burchhardt, G.; Herrmann, H.; Fuchs, G. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol. Rev.* **1999**, *22*, 439−458.
(37) Beller, H. R. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring in situ BTEX biodegradation. *Environ. Sci. Technol.* **2002**, *36*, 2724−2728.
(38) Weissbach, H.; Redfield, B. G.; Dickerman, H.; Brot, N. Studies on methionine biosynthesis. Effect of alkylcobamide derivatives on the formation of holoenzyme. *J. Biol. Chem.* **1965**, *240*, 856−862.
(39) Wood, J. M.; Wolfe, R. S. Propylation and purification of a B12 enzyme involved in methane formation. *Biochemistry* **1966**, *5*, 3598−3603.
(40) Flesher, J. W.; Myers, S. R. Methyl-substitution of benzene and toluene in preparations of human bone marrow. *Life Sci.* **1991**, *48*, 843−850.
(41) Londry, K. L.; Fedorak, P. M. Benzoic-acid intermediates in the anaerobic biodegradation of phenols. *Can. J. Microbiol.* **1992**, *38*, 1−11.
(42) Flyvbjerg, J.; Arvin, E.; Jensen, B. K.; Olsen, S. K. Microbial degradation of phenols and aromatic hydrocarbons in creosote-contaminated groundwater under nitrate-reducing conditions. *J. Contam. Hydrol.* **1993**, *12*, 133−150.
(43) Breinig, S.; Schiltz, E.; Fuchs, G. Genes involved in anaerobic metabolism of phenol in the bacterium *Thauera aromatica*. *J. Bacteriol.* **2000**, *182*, 5849−5863.
(44) Lovley, D. R.; Lonergan, D. J. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. *Appl. Environ. Microbiol.* **1990**, *56*, 1858−1864.
(45) Haggblom, M. M.; Young, L. Y. Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. *Appl. Environ. Microbiol.* **1995**, *61*, 1546−1550.
(46) Bak, F.; Widdel, F. Anaerobic degradation of phenol and phenol derivatives of *Desulfobacterium* phenolicum sp. nov. *Arch. Microbiol.* **1986**, *146*, 177−180.
(47) Bisaillon, J. G.; Beaudet, R.; Lepine, F.; Sylvestre, M. Microbiological study of the carboxylation of phenols by methanogenic fermentation a summary. *Water Pollut. Res. J. Can.* **1994**, *29*, 117−127.
(48) Tao, Y.; Fishman, A.; Bentley, W. E.; Wood, T. K. Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of Pseudomonas mendocina KR1 and toluene 3-monooxygenase of Ralstonia pickettii PKO1. *Appl. Environ. Microbiol.* **2004**, *70*, 3814−3820.

Received for review February 13, 2005. Revised manuscript received May 30, 2005. Accepted June 6, 2005.

ES050294U